Exhibit 3

ARATION OF PAUL POLAKIS, Ph.D.

I, P h.D., declare and say as follows:

1. I we Ph.D. by the Department of Biochemistry of the Michigan State Univ 4. My scientific Curriculum Vitae is attached to and forms part of this (Exhibit A).

2. I am ployed by Genentech, Inc. where my job title is Staff Scientist. enentech in 1999, one of my primary responsibilities has been leadin 's Tumor Antigen Project, which is a large research project with a prin identifying tumor cell markers that find use as targets for both the di reatment of cancer in humans.

3. As p or Antigen Project, my laboratory has been analyzing different arious genes in tumor cells relative to normal cells. The pur is to identify proteins that are abundantly expressed on certain that are either (i) not expressed, or (ii) expressed at lower leve ing normal cells. We call such differentially expressed proteins "a teins". When such a tumor antigen protein is identified, c an antibody that recognizes and binds to that protein. Such an ant in the diagnosis of human cancer and may ultimately serve as utic in the treatment of human cancer.

4. I search conducted by Genentech's Tumor Antigen Project, variety of scientific techniques for detecting and studying pression in human tumor cells relative to normal cells, at genom d protein levels. An important example of one such techniq and widely used technique of microarray analysis which h mely useful for the identification of mRNA molecules that are sed in one tissue or cell type relative to another. In the course microarray analysis, we have identified approxim scripts that are present in human tumor cells at significa in corresponding normal human cells. To date, we have ge ra t bind to about 30 of the tumor antigen proteins expresse ially expressed gene transcripts and have used these antibod etermine the level of production of these tumor antigen n cancer cells and corresponding normal cells. We have the s of mRNA and protein in both the tumor and normal cells an

5. F rotein expression analyses described in paragraph 4 above, w here is a strong correlation between changes in the level of particular cell type and the level of protein

expres... in that cell type. In approximately 80% of our
observ... l that increases in the level of a particular mRNA
correla... n the level of protein expressed from that mRNA when
human t... mpared with their corresponding normal cells.

6. ... experience accumulated in more than 20 years of
researc... discussed in paragraphs 4 and 5 above and my
knowl... cientific literature, it is my considered scientific
opinio... s, an increased level of mRNA in a tumor cell relative
to a no... correlates to a similar increase in abundance of the
encode... r cell relative to the normal cell. In fact, it remains a
central ... biology that increased mRNA levels are predictive of
corresp... els of the encoded protein. While there have been
publish... or which such a correlation does not exist, it is my
opinio... exceptions to the commonly understood general rule
that inc... are predictive of corresponding increased levels of the
encode...

7. ... ll statements made herein of my own knowledge are
true an... ade on information or belief are believed to be true,
and fur... nts were made with the knowledge that willful false
statem... de are punishable by fine or imprisonment, or both,
under ... 3 of the United States Code and that such willful
stateme... e validity of the application or any patent issued
thereon...

Dated: 5/...

By: Paul Polakis

Paul Polakis, Ph.D.

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URRICULUM VITAE

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1997 -1999 — Research Director
Onyx Pharmaceuticals, Richmond, CA

1992- 1996 — Senior Scientist, Project Leader, Onyx
Pharmaceuticals, Richmond, CA

1991-1992 — Senior Scientist, Chiron Corporation,
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1987-1989 — Postdoctoral Research Associate, Genentech,
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1984-1985 — Assistant Professor, Department of Chemistry, Oberlin College, Oberlin, Ohio

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cancer tissues.

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Torben F. Ø... ...ft‡§, Th...m... ...rǁ, Frederic M. Waldmanǁ, Hans Wolf**, and Julio E. ...lis‡‡

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phenomenon at both the transcription and translation levels. High throughput array studies of the breast cancer cell line BT474 has suggested that there is a correlation between DNA copy numbers and gene expression in highly amplified areas (2), and studies of individual genes in solid tumors have revealed a good correlation between gene dose and mRNA or protein levels in the case of c-erb-B2, *cyclin d1*, *ems1*, and N-myc (3–5). However, a high cyclin D1 protein expression has been observed without simultaneous amplification (4), and a low level of c-myc copy number increase was observed without concomitant c-myc protein overexpression (6).

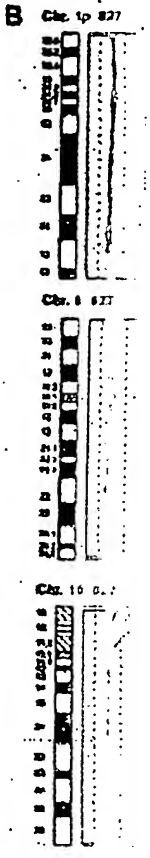
In human bladder tumors, karyotyping, fluorescent *in situ* hybridization, and comparative genomic hybridization (CGH)[1] have revealed chromosomal aberrations that seem to be characteristic of certain stages of disease progression. In the case of non-invasive pTa transitional cell carcinomas (TCCs), this includes loss of chromosome 9 or parts of it, as well as loss of Y in males. In minimally invasive pT1 TCCs, the following alterations have been reported: 2q−, 11p−, 1q+, 11q13+, 17q+, and 20q+ (7–12). It has been suggested that these regions harbor tumor suppressor genes and oncogenes; however, the large chromosomal areas involved often contain many genes, making meaningful predictions of the functional consequences of losses and gains very difficult.

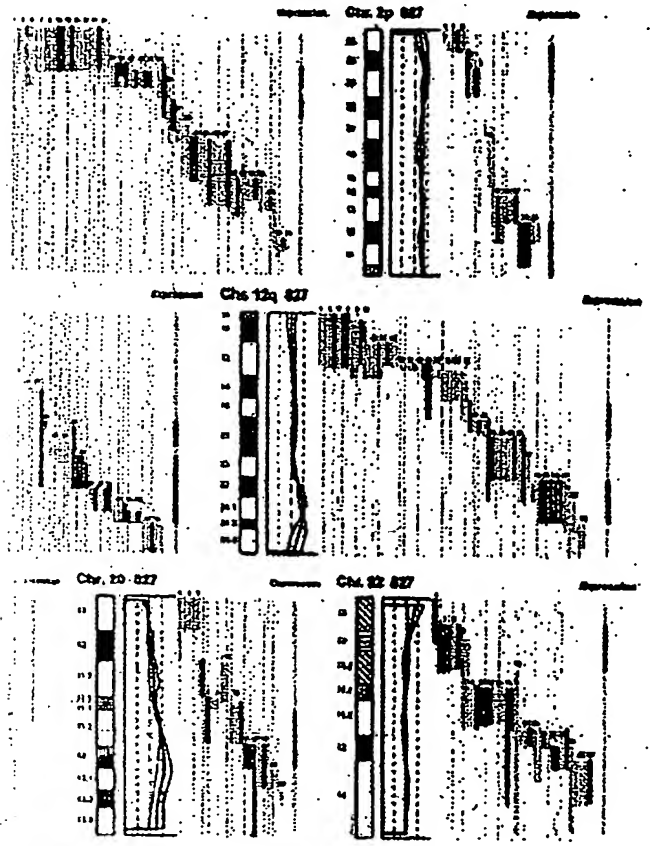
In this investigation we have combined genome-wide technology for detecting genomic gains and losses (CGH) with gene expression profiling techniques (microarrays and proteomics) to determine the effect of gene copy number on transcript and protein levels in pairs of non-invasive and invasive human bladder TCCs.

EXPERIMENTAL PROCEDURES

Material—Bladder tumor biopsies were sampled after informed consent was obtained and after removal of tissue for routine pathology examination. By light microscopy tumors 335 and 532 were staged by an experienced pathologist as pTa (superficial papillary),

[1] The abbreviations used are: CGH, comparative genomic hybridization; TCC, transitional cell carcinoma; LOH, loss of heterozygosity; PA-FABP, psoriasis-associated fatty acid-binding protein; 2D, two-dimensional.





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labeled reference DNA (200 ng), and human Cot-1 DNA (20 μg) were denatured at 37 °C for 5 min and applied to denatured normal metaphase slides. Hybridization was at 37 °C for 2 days. After washing, the slides were counterstained with 0.15 μg/ml 4,6-diamidino-2-phenylindole in an anti-fade solution. A second hybridization was performed for all tumor samples using fluorescein-labeled reference DNA and Texas Red-labeled tumor DNA (inverse labeling) to confirm the aberrations detected during the initial hybridization. Each CGH experiment also included a normal control hybridization using fluorescein- and Texas Red-labeled normal DNA. Digital image analysis was used to identify chromosomal regions with abnormal fluorescence ratios, indicating regions of DNA gains and losses. The average green:red fluorescence intensity ratio profiles were calculated using four images of each chromosome (eight chromosomes total) with normalization of the green:red fluorescence intensity ratio for the entire metaphase and background correction. Chromosome identification was performed based on 4,6-diamidino-2-phenylindole banding patterns. Only images showing uniform high intensity fluorescence with minimal background staining were analyzed. All centromeres, p arms of acrocentric chromosomes, and heterochromatic regions were excluded from the analysis.

RESULTS

Comparative Genomic Hybridization—The CGH analysis identified a number of chromosomal gains and losses in the

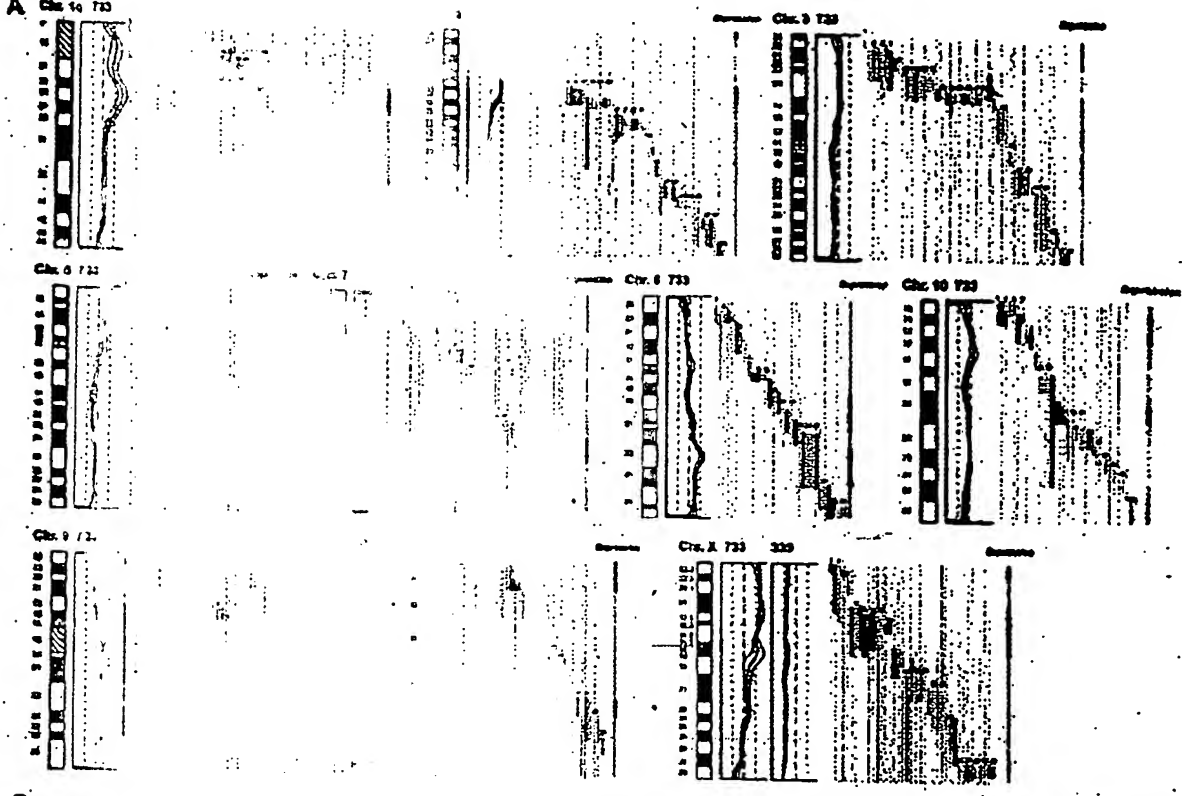

FIG. 1. ... expression ... compared ... counterpart ... (left). The ... deviation ... 0.5 (left) an... profile of ... running num... the gene, a... increase (b... in expressi... down-regul... another. It ... determinat...

...om left to right are chromosome (Chr.), CGH profiles, gene location and ...ong the chromosome. A, expression of mRNA in invasive tumor 733 as ...ession of mRNA in invasive tumor 827 compared with the non-invasive ...n tumor DNA and normal DNA is shown along the length of the chromosome ...chromosomes and is surrounded by thin curves indicating one standard ...1 (no change), and the vertical lines next to it (dotted) indicate a ratio of ...335 used for comparison showed alterations in DNA content, the ratio ...or profile. The colored bars represents one gene each, identified by the ...t www.MDL.DK/sdata.html). The bars indicate the purported location of ...the invasive tumor compared with the non-invasive counterpart; >2-fold ...e). The bar to the far right, entitled Expression shows the resulting change ...t half of the genes were up-regulated (black), at least half of the genes ...d (orange). If a gene was absent in one of the samples and present in ...of was chosen as this corresponded to one standard deviation in a double ...regions were excluded from data analysis.

grade I and ... (invasive ... staged ac... mRNA ... were embe... solution a... RNAzol B ... poly(A)+ fr... mRNA kit; ... cRNA Pro... The first and ... SuperScript ... turer's instr... polymerase ... GAscript ...

UTP (Enzo) was used, together with unlabeled NTPs in the reaction. Following the *In vitro* transcription reaction, the unincorporated nucleotides were removed using RNeasy columns (Qiagen).

Array Hybridization and Scanning—Array hybridization and scanning was modified from a previous method (13). 10 μg of cRNA was fragmented at 94 °C for 35 min in buffer containing 40 mM Tris acetate, pH 8.1, 100 mM KOAc, 30 mM MgOAc. Prior to hybridization, the fragmented cRNA in a 6× SSPE-T hybridization buffer (1 M NaCl, 10 mM Tris, pH 7.6, 0.005% Triton), was heated to 95 °C for 5 min, subsequently cooled to 40 °C, and loaded onto the Affymetrix probe array cartridge. The probe array was then incubated for 16 h at 40 °C at constant rotation (60 rpm). The probe array was exposed to 10 washes in 6× SSPE-T at 25 °C followed by 4 washes in 0.5× SSPE-T at 50 °C. The biotinylated cRNA was stained with a streptavidin-phycoerythrin conjugate, 10 μg/ml (Molecular Probes) in 6× SSPE-T

TABLE I

Correlation [illegible] alterations [illegible] by CGH and by expression monitoring

Top, CGH [illegible] independent [illegible] (CGH alterations — what expression ratio was found); bottom, altered expression used as independent [illegible] what CGH alteration was found).

CGH altera[illegible]	733 [illegible]	[illegible]
13 G[illegible]		
10 Los[illegible]	[illegible]-regulation; [illegible]-regulati[illegible]; [illegible]	[illegible]

Expression c[illegible]	[illegible]	[illegible]
16 Up-[illegible]	[illegible]	
21 Down-[illegible]		
15 No c[illegible]	[illegible] Gain; [illegible]os[illegible]	

CGH alterations	Tumor 827 vs. 532 Expression change clusters	Concordance
10 Gain	8 Up-regulation 0 Down-regulation 2 No change	80%
12 Loss	3 Up-regulation 2 Down regulation 7 No change	17%

Expression change clusters	Tumor 827 vs. 532 CGH alterations	Concordance
17 Up-regulation	10 Gain 5 Loss 2 No change	59%
9 Down-regulation	0 Gain 3 Loss 6 No change	33%
21 No change	1 Gain 3 Loss 17 No change	81%

two invasi[illegible] the two no[illegible] 532) show[illegible] and Y−, res[illegible] (1q22-24+, [illegible]14.[illegible] 9q34+, 11[illegible] 13+ [illegible] ical for thei[illegible] some of [illegible] losses devi[illegible] and the a[illegible] In the cas[illegible] changes, content, [illegible] 20q12 in T[illegible] *mRNA* [illegible] mRNA lev[illegible] 733) were (TCCs 53[illegible] ments in [illegible] 532, res[illegible] arrays to proximat[illegible] were sea[illegible] chromoso[illegible] (1096) w[illegible] that way [illegible] DNA cop[illegible] vidual ch[illegible]

For ea[illegible] w[illegible] the invasi[illegible] r[illegible] represen[illegible] accordin[illegible]

than 2-fold were regarded as informative (Fig. 1). The density of genes along the chromosomes varied, and areas containing only one gene were excluded from the calculations. The resolution of the CGH method is very low, and some of the outlier data may be because of the fact that the boundaries of the chromosomal aberrations are not known at high resolution.

Two sets of calculations were made from the data. For the first set we used CGH alterations as the independent variable and estimated the frequency of expression alterations in these chromosomal areas. In general, areas with a strong gain of chromosomal material contained a cluster of genes having increased mRNA expression. For example, both chromosomes 1q21-q25, 2p and 9q, showed a relative gain of more than 100% in DNA copy number that was accompanied by increased mRNA expression levels in the two tumor pairs (Fig. 1). In most cases, chromosomal gains detected by CGH were accompanied by an increased level of transcripts in both TCCs 733 (77%) and 827 (80%) (Table I, *top*). Chromosomal losses, on the other hand, were not accompanied by decreased expression in several cases, and were often registered as having unaltered RNA levels (Table I, *top*). The inability to detect RNA expression changes in these cases was not because of fewer genes mapping to the lost regions (data not shown).

In the second set of calculations we selected expression alterations above 2-fold as the independent variable and estimated the frequency of CGH alterations in these areas. As above, we found that increased transcript expression correlated with gain of chromosomal material (TCC 733, 69% and TCC 827, 59%), whereas reduced expression was often detected in areas with unaltered CGH ratios (Table I, *bottom*). Furthermore, as a control we looked at areas with no alter-

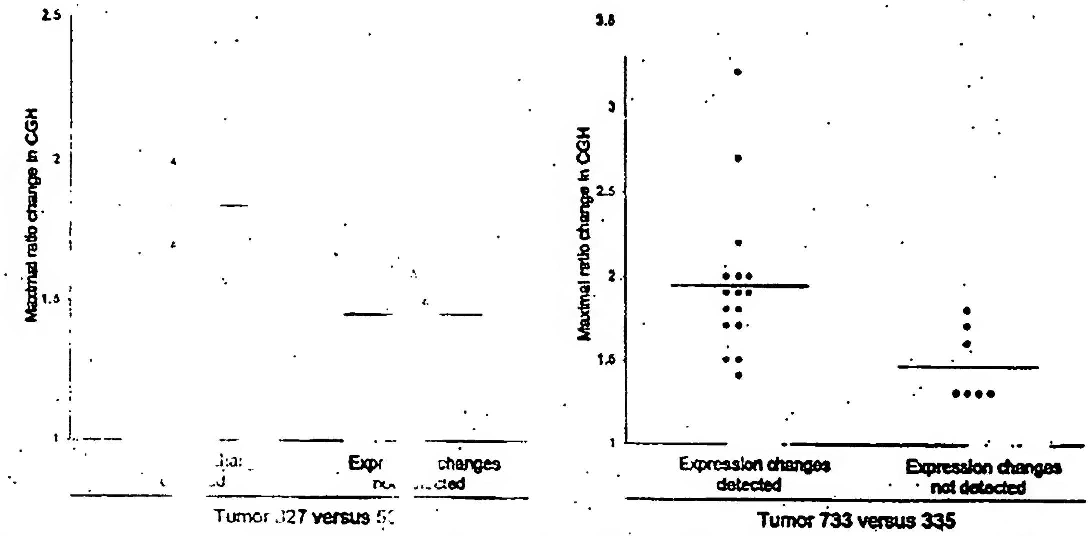

FIG. 2. Correlation ... en maximum CGH ... ration and the ability to detect expression change by oligonucleotide array monitoring. The abs... shown as a numeric... change in ratio between invasive tumors 827 (▲) and 733 (◆) and their non-invasive counterparts 532 and ... expression change ... taken from the *Expression* line to the *right* in Fig. 1, which depicts the resulting expression change to ... somal region ... least half of the mRNAs from a given region have to be either up- or down-regulated to be scored as an ... All chromo... arms in which the CGH ratio plus or minus one standard deviation was outside the ratio value of one ... ed.

ation in expression. ... most of these areas ... Table I, *bottom*). ... increased ... DNA ... somal area ... changes, ... regions sho... change in ... tide arrays ... TCC 827 ... observed b... the DNA co... based tec... areas with ... ables. The... ratio devia... not at low... loss of an ... sion level, ... sion altera... much larg...

Microsat... es—In TCC ... amplificatio... mal CGH ... chromoso... 10q22). To undetecte...

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because of other non-structural mechanisms regulating transcription, we examined two microsatellites positioned at chromosome 1q25–32 and two at chromosome 2p22. Loss of heterozygosity (LOH) was found at both 1q25 and at 2p22 indicating that minor deleted areas were not detected with the resolution of CGH (Fig. 3). Additionally, chromosome 2p in TCC 733 showed a CGH pattern of gain/no change/gain of DNA that correlated with transcript increase/decrease/increase. Thus, for the areas showing increased expression there was a correlation with the DNA copy number alterations (Fig. 1*A*). As indicated above, the mRNA decrease observed in the middle of the chromosomal gain was because of LOH, implying that one of the mechanisms for mRNA down-regulation may be regions that have undergone smaller losses of chromosomal material. However, this cannot be detected with the resolution of the CGH method.

In both TCC 733 and TCC 827, the telomeric end of chromosome 11p showed a normal ratio in the CGH analysis; however, clusters of five and three genes, respectively, lost their expression. Two microsatellites (D11S1760, D11S922) positioned close to MUC2, IGF2, and cathepsin D indicated LOH as the most likely mechanism behind the loss of expression (data not shown).

A reduced expression of mRNA observed in TCC 733 at chromosomes 3q24, 11p11, 12p12.2, 12q21.1, and 16q24 and in TCC 827 at chromosome 11p15.5, 12p11, 15q11.2, and 18q12 was also examined for chromosomal losses using microsatellites positioned as close as possible to the gene loci

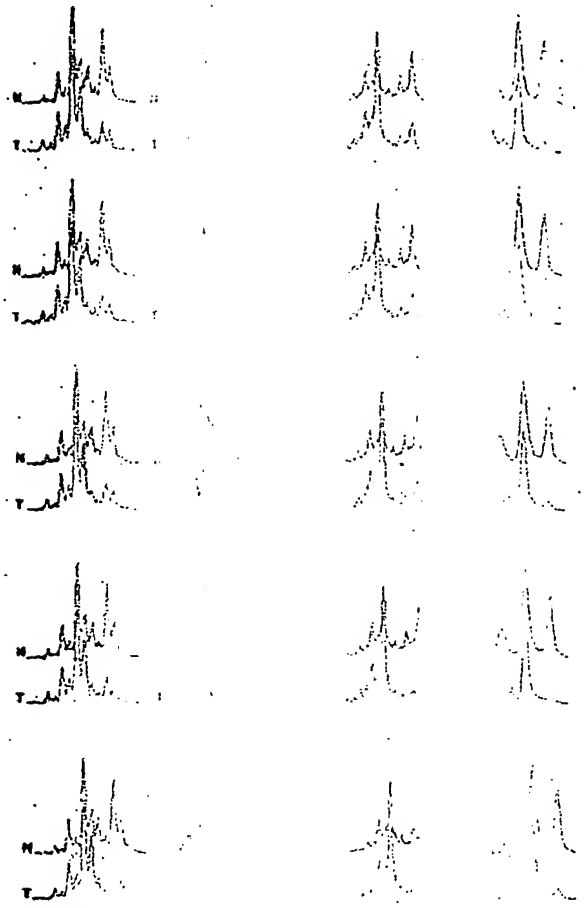

FIG. 3. Mi... ...733 showing ... (a) by DISC... number 38 i... number 41 in ... to general β ... showing lo... close to mit... 12 in Fig. 1; ... from norm... electrophere... partially los...

showing r... positioned ... transcripti... may be c... *Relation* ... 2D-PAGE ... Blue and/... using fres... proteins ...

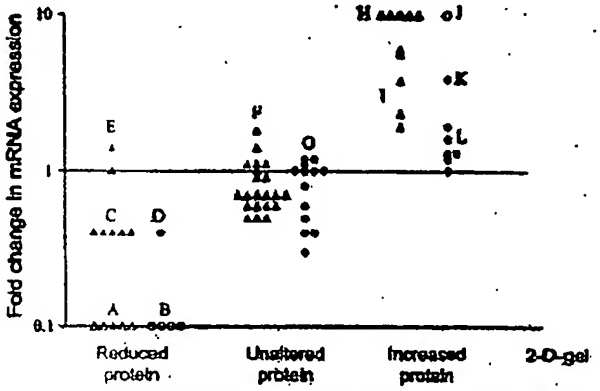

FIG. 4. Correlation between protein levels as judged by 2D-PAGE and transcript ratio. For comparison proteins were divided in three groups, unaltered in level or up- or down-regulated (*horizontal axis*). The mRNA ratio as determined by oligonucleotide arrays was plotted for each gene (*vertical axis*). ▲, mRNAs that were scored as present in both tumors used for the ratio calculation; Δ, mRNAs that were scored as absent in the invasive tumors (along *horizontal axis*) or as absent in non-invasive reference (*top* of figure). Two different scalings were used to exclude scaling as a confounder. TCCs 827 and 532 (▲Δ) were scaled with background suppression, and TCCs 733 and 335 (●○) were scaled without suppression. Both comparisons showed highly significant ($p < 0.005$) differences in mRNA ratios between the groups. Proteins shown were as follows: Group *A* (from *left*), phosphoglucomutase 1, glutathione transferase class-μ number 4, fatty acid-binding protein homologue, cytokeratin 15, and cytokeratin 13; *B* (from *left*), fatty acid-binding protein homologue, 28-kDa heat shock protein, cytokeratin 13, and calcyclin; *C* (from *left*), α-enolase, hnRNP B1, 28-kDa heat shock protein, 14-3-3-ε, and pre-mRNA splicing factor; *D*, mesothelial keratin K7 (type II); *E* (from *top*), glutathione *S*-transferase-π and mesothelial keratin K7 (type II); *F* (from *top* and *left*), adenylyl cyclase-associated protein, E-cadherin, keratin 19, calgizzarin, phosphoglycerate mutase, annexin IV, cytoskeletal γ-actin, hnRNP A1, integral membrane protein calnexin (IP90), hnRNP H, brain-type clathrin light chain-a, hnRNP F, 70-kDa heat shock protein, heterogeneous nuclear ribonucleoprotein A/B, translationally controlled tumor protein, liver glyceraldehyde-3-phosphate dehydrogenase, keratin 8, aldehyde reductase, and Na,K-ATPase β-1 subunit; *G*, (from *top* and *left*), TCP20, calgizzarin, 70-kDa heat shock protein, calnexin, hnRNP H, cytokeratin 15, ATP synthase, keratin 19, triosephosphate isomerase, hnRNP F, liver glyceraldehyde-3-phosphatase dehydrogenase, glutathione *S*-transferase-π, and keratin 8; *H* (from *left*), plasma gelsolin, autoantigen calreticulin, thioredoxin, and NAD+-dependent 15 hydroxyprostaglandin dehydrogenase; *I* (from *top*), prolyl 4-hydroxylase β-subunit, cytokeratin 20, cytokeratin 17, prohibitin, and fructose 1,6-biphosphatase; *J* annexin II; *K*, annexin IV; *L* (from *top* and *left*), 90-kDa heat shock protein, prolyl 4-hydroxylase β-subunit, α-enolase, GRP 78, cyclophilin, and cofilin.

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gradient, and having a known chromosomal location, were selected for analysis in the TCC pair 827/532. Proteins were identified by a combination of methods (see "Experimental Procedures"). In general there was a highly significant correlation ($p < 0.005$) between mRNA and protein alterations (Fig. 4). Only one gene showed disagreement between transcript alteration and protein alteration. Except for a group of cyto-

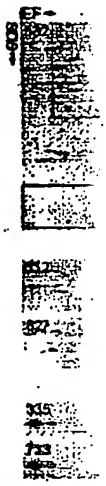

Fig. 5. C
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keratins encoded by genes on chromosome 17 (Fig. 5) the analyzed proteins did not belong to a particular family. 28 well focused proteins whose genes had a know chromosomal location were detected in TCCs 733 and 335, and of these 19 correlated ($p < 0.005$) with the mRNA changes detected using the arrays (Fig. 4). For example, PA-FABP was highly expressed in the non-invasive TCC 335 but lost in the invasive counterpart (TCC 733; see Fig. 5). The smaller number of proteins detected in both 733 and 335 was because of the smaller size of the biopsies that were available.

11 chromosomal regions where CGH showed aberrations that corresponded to the changes in transcript levels also showed corresponding changes in the protein level (Table II). These regions included genes that encode proteins that are found to be frequently altered in bladder cancer, namely cytokeratins 17 and 20, annexins II and IV, and the fatty acid-binding proteins PA-FABP and FBP1. Four of these proteins were encoded by genes in chromosome 17q, a frequently amplified chromosomal area in invasive bladder cancers.

DISCUSSION

Most human cancers have abnormal DNA content, having lost some chromosomal parts and gained others. The present study provides some evidence as to the effect of these gains and losses on gene expression in two pairs of non-invasive and invasive TCCs using high throughput expression arrays and proteomics, in combination with CGH. In general, the results showed that there is a clear individual regulation of the mRNA expression of single genes, which in some cases was superimposed by a DNA copy number effect. In most cases, genes located in chromosomal areas with gains often exhibited increased mRNA expression, whereas areas showing losses showed either no change or a reduced mRNA expression. The latter might be because of the fact that losses most often are restricted to loss of one allele, and the cut-off point for detection of expression alterations was a 2-fold change, thus resulting at the border of detection. In several cases, how-

TABLE II
Genes with both mRNA and gene dose changes

TCC	CGH alteration	Transcript alteration[a]	Protein alteration
	Gain	Abs to Pres[a]	Increase
	Gain	3.9-Fold up	Increase
	Gain	3.8-Fold up	Increase
	Gain	5.6-Fold up	Increase
	Loss	10-Fold down	Decrease
	Gain	2.3-Fold up	Increase
	Gain	Abs to Pres	Increase
	Loss	2.5-Fold up	Decrease
	Gain	3.7-/2.5-Fold up[b]	Increase
	Gain	6.7-/1.8-Fold up	Increase
	Loss	2.5-Fold down	Decrease

[b] In TCCs 827 and 733 these are shown as 827/733.

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...arm and that the use of cDNA microarrays for analysis of DNA copy number changes will reach a resolution that can resolve these changes, as has recently been proposed (2). The outlier data were not more frequent at the boundaries of the CGH aberrations. At present we do not know the mechanism behind chromosomal aneuploidy and cannot predict whether chromosomal gains will be transcribed to a larger extent than the two native alleles. A mechanism as genetic imprinting has an impact on the expression level in normal cells and is often reduced in tumors. However, the relation between imprinting and gain of chromosomal material is not known.

We regard it as a strength of this investigation that we were able to compare invasive tumors to benign tumors rather than to normal urothelium, as the tumors studied were biologically very close and probably may represent successive steps in the progression of bladder cancer. Despite the limited amount of fresh tissue available it was possible to apply three different state of the art methods. The observed correlation between ... number and mRNA expression is remarkable when ... numbers that different pieces of the tumor biopsies were ... the different sets of experiments. This indicate that ... tumors are relatively homogenous, a notion recently ... by CGH and LOH data that showed a remarkable ... even between tumors and distant metastasis (10, 23).

... few cases analyzed, mRNA and protein levels ... a striking correspondence although in some cases ... discrepancies that may be attributed to translational ... post-translational processing, protein degradation ... combination of these. Some transcripts belong to ... mRNA pools, which are associated with few ... inactive ribosomes; these pools, however, ... rare (24). Protein degradation, for example, may ... important in the case of polypeptides with a short ... (e.g. signaling proteins). A poor correlation between ... protein levels was found in liver cells as deter... ... arrays and 2D-PAGE (25), and a moderate correla... ... recently reported by Ideker *et al.* (26) in yeast.

... Surprisingly, our study revealed a much better correlation ... gained chromosomal areas and increased mRNA ... between loss of chromosomal areas and reduced ... els. In general, the level of CGH change determined ... to detect a change in transcript. One possible ...tion could be that by losing one allele the change in ...vel is not so dramatic as compared with gain of ..., which can be rather unlimited and may lead to a ...d increase in gene copy number resulting in a much ...pact on transcript level. The latter would be much ... detect on the expression arrays as the cut-off point ...d at a 2-fold level so as not to be biased by noise on ... Construction of arrays with a better signal to noise ... In the future allow detection of lesser than 2-fold ... in transcript levels, a feature that may facilitate the ... of the effect of loss of chromosomal areas on tran... ...vels.

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ABSTRACT

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INTRODUCTI

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Patterns in Breast Cancer[1,2]

, Maija Wolf, Spyro Mousses, Ester Rozenblum,
, Olli-P. Kallioniemi, and Anne Kallioniemi[4]

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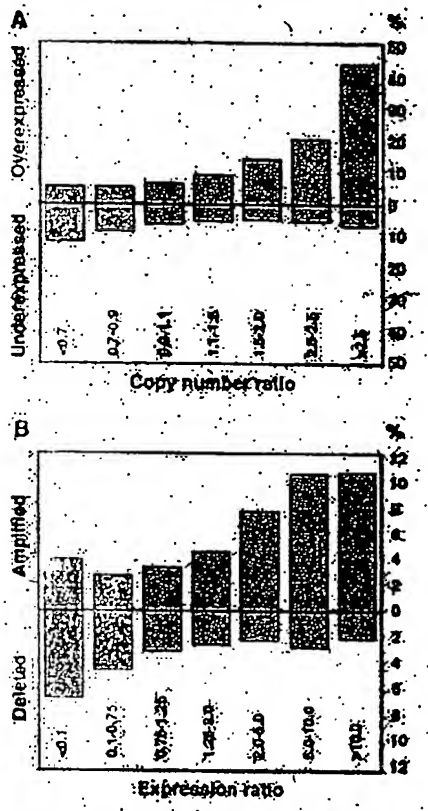
and Biomedicum Biochip Center, Helsinki University Hospital, Biomedicum

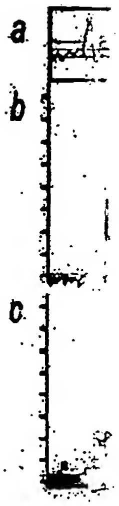


Impact of copy number on global gene expression levels. *A*, percentage of underexpressed genes (*Y axis*) according to copy number ratios (*X axis*). Ratios used to define over- and underexpression were >2.184 (global upper 7% of ratios) and <0.4825 (global lower 7% of the expression ratios). *B*, percentage amplified and deleted genes according to expression ratios. Threshold values for amplification and deletion were >1.5 and <0.7.

recurrent regions of DNA amplification have been mapped in cancer by CGH[5] (9, 10). However, these amplicons are often poorly defined, and their impact on gene expression remains

hesized that genome-wide identification of those gene changes that are attributable to underlying gene copy rations would highlight transcripts that are actively in- the causation or maintenance of the malignant phenotype. such transcripts, we applied a combination of cDNA and roarrays to: (*a*) determine the global impact that gene copy ation plays in breast cancer development and progression; ntify and characterize those genes whose mRNA expres-

[5] Abbreviations used: CGH, comparative genomic hybridization; FISH, fluorescence in situ hybridization; RT-PCR, reverse transcription-PCR.



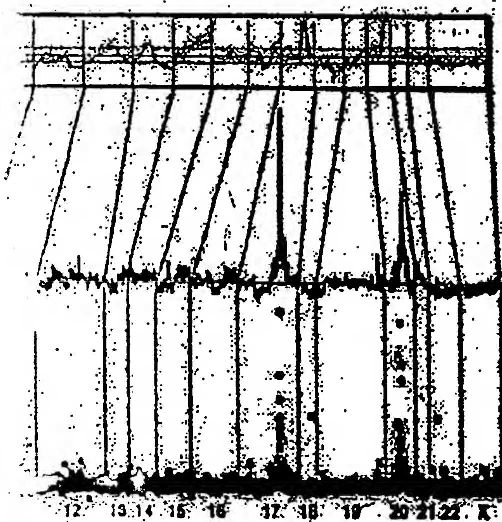


Fig. 2. Genomic ... *line*) across the entir... and *green line*, a ratio ... of the cDNA clones at... copy number ratio of 1... the next 5% of the ex... (underexpressed genes); indicated with a *dashe*... ...mosomal CGH analysis of MCF-7. The copy number ratio profile (*blue* ... The *black horizontal line* indicates a ratio of 1.0; *red line*, a ratio of 0.8; ...array. The copy number ratios were plotted as a function of the position ... moving median of 10 adjacent clones is shown. *Red horizontal line*, the ...ssion ratios. The *bright red dots* indicate the upper 2%, and *dark red dots*, ... the lowest 2%, and *dark green dots*, the next 5% of the expression ratios ...nb rs are shown at the *bottom* of the figure, and chromosome boundaries are

sion is most si... sponding geno...

MATERIALS A...

Breast Cancer C... 474, HCC1428, H... SKBR-3, T-47D, ... American Type C... recommended cu... using standard p...

Copy Numb... preparation and ... performed as desc... terized expressed ... genes. CGH expe... 15). Briefly, 20 μ... human WBCs we... ogies, Inc., Rockv... μg of digested ... Pharmacia) and ... the Bioprime La... posthybridization analyses, a stand... La Jolla, CA) was ... labeled with Cy... labeled cDNAs w... microarray analy... Alto, CA) was ... locations using ... average intensiti... average intensity ... the copy numb... distribution of ... on the basis of 85 ... array. Low quali... intensity <100 ... reference intensi...

...d from the analysis and were treated as missing values. The ...f fluorescence ratios were used to define cutpoints for increased/...y number. Genes with CGH ratio >1.43 (representing the upper ...l ratios across all experiments) were considered to be amplified, ...h ratio <0.73 (representing the lower 5%) were considered to be

... **Analysis of CGH and cDNA Microarray Data.** To evaluate ...ct of copy number alterations on gene expression, we applied the ...tistical approach. CGH and cDNA calibrated intensity ratios were ...d and normalized using median centering of the values in each ...ermore, cDNA ratios for each gene across all 14 cell lines were ...l. For each gene, the CGH data were represented by a vector ...d 1 for a...plification (ratio, >1.43) and 0 for no amplification, ...was correlated with gene expression using the signal-to-noise ...We calculated a weight, w_g, for each gene as follows:

$$w_g = \frac{m_{g1} - m_{g0}}{\sigma_{g1} + \sigma_{g0}}$$

... and m_{g0}, ... σ_{g0} denote the means and SDs for the expression ...lified ar... nonamplified cell lines, respectively. To assess the ...ificance of each weight, we performed 10,000 random permu-...label vec... The probability that a gene had a larger or equal ...om permutation than the original weight was denoted by α. A ... indicates a strong association between gene expression and

... **Localization of cDNA Clones and Amplicon Mapping.** Each ...n the microarray was assigned to a Unigene cluster using the ...141.[6] A database of genomic sequence alignment information ...uences was created from the August 2001 freeze of the Uni-...ruis S... Cruz's GoldenPath database.[7] The chromosome and ...each c...NA clone were then retrieved by relating these data ...were d... as a CGH copy number ratio >2.0 in at least two ...in two ...re cell lines or a CGH ratio >2.0 in at least three ...in a single cell line. The amplicon start and end positions were

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...ess: http://research.nhgri.nih.gov/microarray/downloadable_cdna.html.
...ess: www.genome.ucsc.edu.

Table 1 *Summ*

Location
1p13
1q21
1q22
3p14
7p12.1–7p11.2
7q31
7q32
8q21.11–8q21.1
8q21.3
8q23.3–8q24.1
8q24.22
9p13
13q22–q31.
16q22
17q11
17q12–q21.2
17q21.32–q21.3
17q22–q23.3
17q23.3–q24.3
19q13
20q11.22
20q13.12
20q13.12–q13.1
20q13.2–q13.32

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RT-PCR. T
GAPDH. Reve
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as a template. *H*
and 5′-GCGTCA

RESULTS

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was 267 kb.
breast cancer
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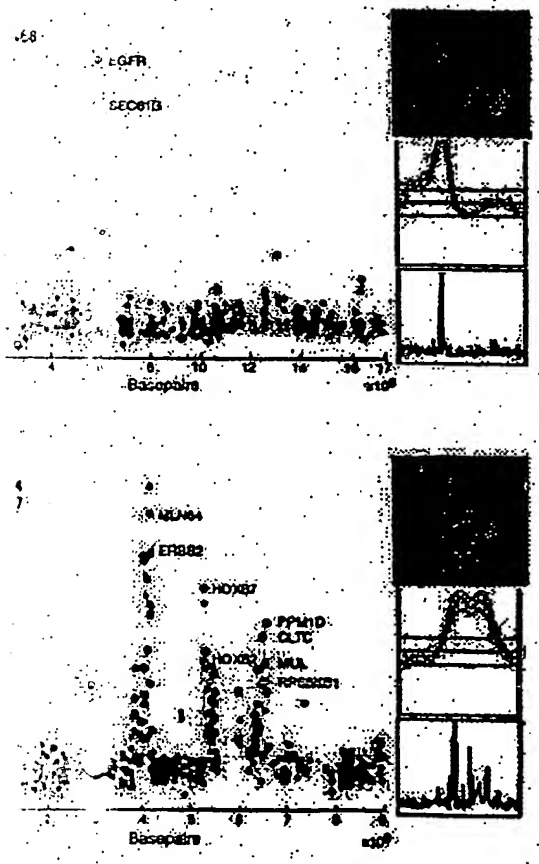
validated, with 1q21, 17q12–q21.2, 17q22–q23, 20q13.1, .2 regions being most commonly amplified. Furthermore, ries of these amplicons were precisely delineated. In ad- el amplicons were identified at 9p13 (38.65–39.25 Mb), .3 (52.47–55.80 Mb).

dentification of Putative Amplification Target Genes. /CGH microarray technique enables the direct correla- y number and expression data on a gene-by-gene basis the genome. We directly annotated high-resolution with gene expression data using color coding. Fig. 2*C* most of the amplified genes in the MCF-7 breast cancer 1p13, 17q22–q23, and 20q13 were highly overex- view of chromosome 7 in the MDA-468 cell line *EGFR* as the most highly overexpressed and amplified 1–p12 (Fig. 3*A*). In BT-474, the two known amplicons nd 17q22–q23 contained numerous highly overex- es (Fig. 3*B*). In addition, several genes, including the genes *HOXB2* and *HOXB7*, were highly amplified in a un lescribed independent amplicon at 17q21.3. *HOXB7* tically a plified (as validated by FISH, Fig. 3*B*, *inset*) verexpre ed (as verified by RT-PCR, data not shown) UACC81 , and ZR-75-30 cells. Furthermore, this novel



tation of gen expression data on CGH microarray profiles. *A*, genes in the con in the M A-468 cell line are highly expressed (*red dots*) and include gene. *B*, se al genes in the 17q12, 17q21.3, and 17q23 amplicons in the cancer cel s are highly overexpressed (*red*) and include the *HOXB7* a labels an color coding are as indicated for Fig. 2*C*. *Insets* show GH profil for the corresponding chromosomes and validation of the number nterphase FISH using *EGFR* (*red*) and chromosome 7 MDA-468 (*A*) and *HOXB7*-specific probe (*red*) and chro- (*green*) to BT-474 cells (*B*).

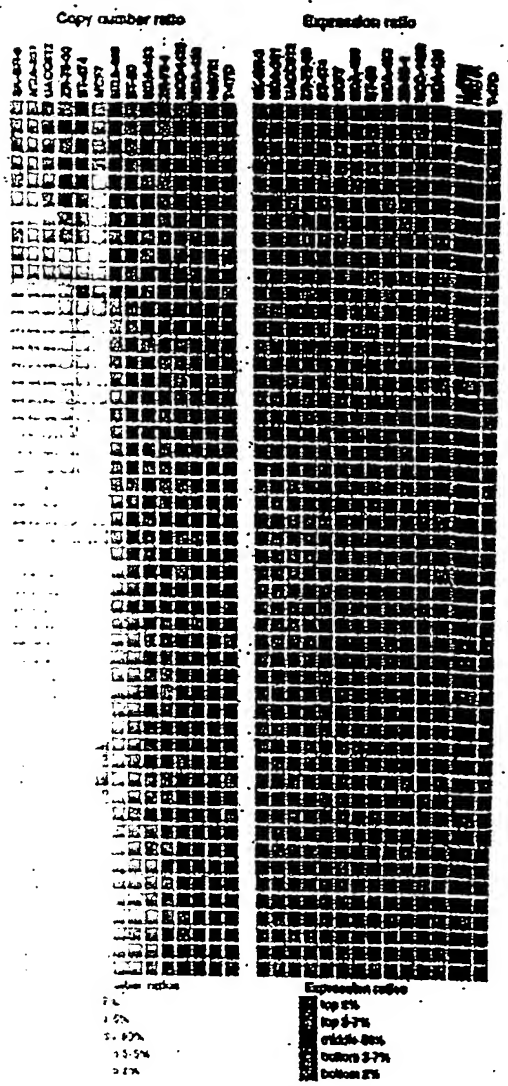


Fig. 4. List of
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[8] Internet addres

urrent gene and chromosome copy number
nt and progression of solid tumors has been
ublications applying CGH[9] (9, 10), as well
her molecular cytogenetic, cytogenetic, and
s. The effects of these somatic genetic
ion levels have remained largely unknown,
ve explored gene expression changes occur-
s (15, 19–21). Here, we applied genome-
to identify transcripts whose expression
to underlying gene copy number alterations

py number on gene expression patterns was
dramatic effects seen in the case of high-

ncbi.nlm.nih.gov/entrez.

level copy numb
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57 amplification and poor patient prognosis. Overall,
ustrate how the identification of genes activated by
tion provides a powerful approach to highlight
important role in cancer as well as to prioritize and
tive targets for therapy development.

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, Stefanie S. Jeffrey††, Per E. Lonning‡‡,
O. Brown†§¶

and †Howard Hughes Medical Institute, Stanford
pital, Montebello, N-0310 Oslo, Norway;
Department of Genetics and Lineberger

we have identified a significant impact of wide-
copy number alteration on the transcriptional
ast tumors.

ethods

Cell Lines. Primary breast tumors were predominantly termediate-grade, infiltrating ductal carcinoma than 50% being lymph node positive. The cells within specimens averaged at least 50%. dual tumors have been published (8, 9), and in Table 1, which is published as supporting the PNAS web site, www.pnas.org. Breast cancer obtained from the American Type Culture mic DNA was isolated either using Qiagen columns, or by phenol/chloroform extraction ol precipitation.

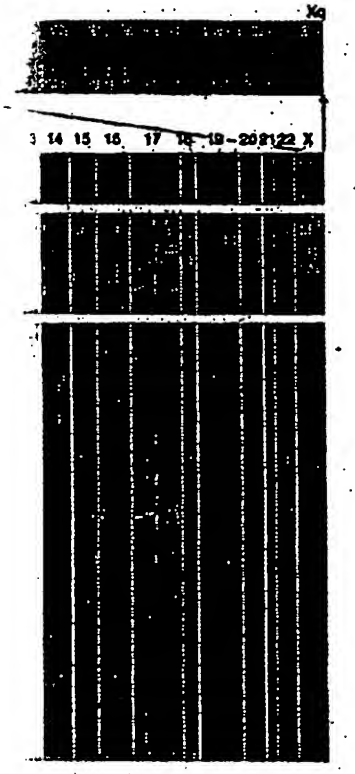
Microarray Hybridizations. Genomic DNA labelions were performed essentially as described 7), with slight modifications. Two micrograms ed in a total volume of 50 microliters and the gents were adjusted accordingly. "Test" DNA cell lines) was fluorescently labeled (Cy5) and human cDNA microarray containing 6,691 human genes (i.e., UniGene clusters). The eled with Cy3) for each hybridization was norte DNA from a single donor. The fabrication arrays and the labeling and hybridization of ve been described (8).

Map Positions. Hybridized arrays were scanned ner (Axon Instruments, Foster City, CA), and (test/reference) calculated using SCANALYZE at http://rana.lbl.gov). Fluorescence ratios for each array by setting the average log for all array elements equal to 0. Measurence intensities more than 20% above backdered reliable. DNA copy number profiles ficantly from background ratios measured in NA control hybridizations were interpreted as NA copy number alteration (see *Estimating ed Fluorescence Ratios* in the supporting indicated, DNA copy number profiles are average (symmetric 5-nearest neighbors), rrayed human cDNAs were assigned by

ve genomic hybridization.

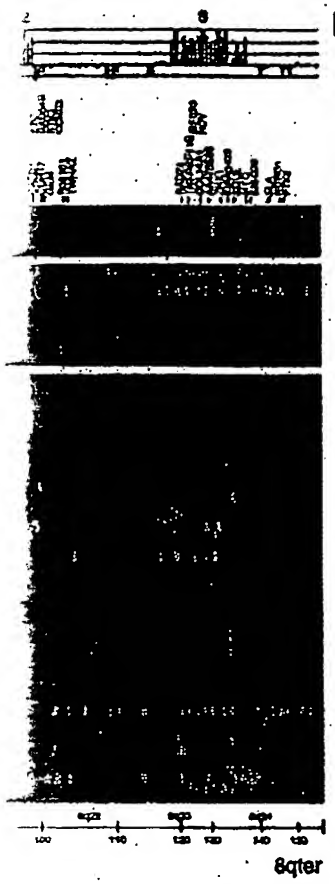
hould be addressed at: Department of Pathology, Stanford
ne, CCSR Building, Room 3245A, 269 Campus Drive, Stanford,
ack1@stanford.edu.

, Hayward, CA 94545.



ofiles are illustrated for cell lines containing different ferent cell line or tumor, and each column represents om 1pter through Xqter. Moving average (symmetric scale (indicated), such that red luminescence reflects en poorly measured data). (*b*) Enlarged view of DNA hromosomes.

nalysis of DNA from cell lines containing f X chromosomes (Fig. 1*b*), as we did before ne sensitivity of our method to detect single-D), and 1.5- (47,XXX), 2- (48,XXXX), or X) gains (also see Fig. 5, which is published nation on the PNAS web site). Fluorescence proportional to copy number ratios, which estimated, in agreement with previous ob-erous DNA copy number alterations were breast cancer cell lines and primary tumors n the tumors despite the presence of euploid s; the magnitudes of the observed changes r in the tumor samples. DNA copy-number nd in every cancer cell line and tumor, and omosome in at least one sample. Recurrent py number gain and loss were readily iden-ic, gains within 1q, 8q, 17q, and 20q were roportion of breast cancer cell lines/tumors 7%, 100%/60%, and 90%/44%, respective- within 1p, 3p, 8p, and 13q (80%/24%, e, and 70%/18%, respectively), consistent genetic studies (refs. 2–4; a complete listing vided in Tables 2 and 3, which are published nation on the PNAS web site). The total



re illustrated for cell lines containing different numbers
rs are separately ordered by hierarchical clustering to
to chromosome 8 are ordered by position along the
lected genes are indicated with color-coded text (red,
d mRNA levels (observed in the majority of the subset
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for breast cancer cell line SKBR3. Fluorescence ratios

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d regions on chromosomes 17 and 20, can be
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a subset of arrayed genes (6,095), mRNA
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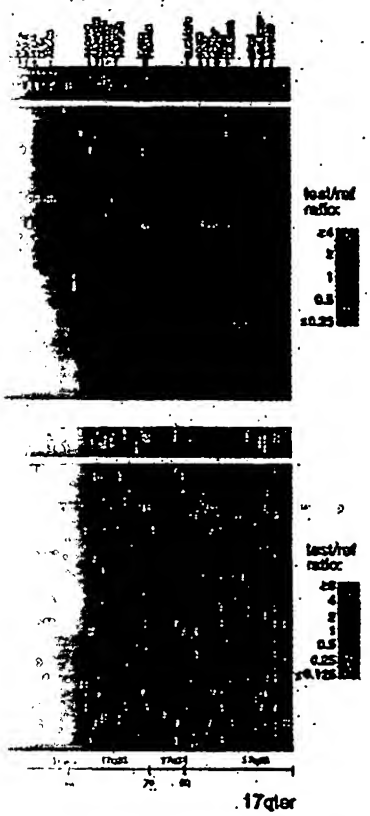
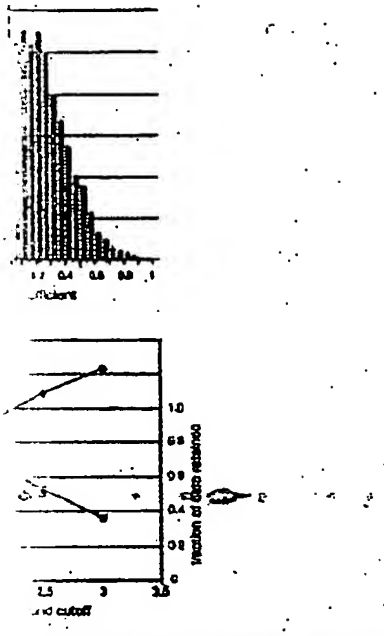


...alteration (*Upper*) and mRNA levels (*Lower*) ... by hierarchical clustering (*Upper*), and the ...ne 17, and for which both DNA copy number ...ated in color-coded text (see Fig. 2 legend).

...ines and tumors, average mRNA levels ...copy number across all five classes, in a ...fashion (*P* values for pair-wise Student's ...ent classes: cell lines, 4×10^{-49}, 1×10^{-49}, ... tumors, 1×10^{-43}, 1×10^{-214}, 5×10^{-41}, ...gression of the average log(DNA copy ...lass, against average log(mRNA level) ...average, a 2-fold change in DNA copy ...ied by 1.4- and 1.5-fold changes in mRNA ...cer cell lines and tumors, respectively (Fig. ...ot shown). Second, we characterized the ...25 correlations between DNA copy num... ...ach across the 37 tumor samples (Fig. 4*b*). ...orrelations forms a normal-shaped curve, ...tedly shifted in the positive direction from ...tically significant, as evidenced in a plot ...ed correlations (Fig. 4*c*), and reflects a ...ite of DNA copy number alterations on ...; the highest correlations between DNA ...A level (the right tail of the distribution ...th amplified and deleted genes (data not ...a linear regression model to estimate the ...measured in mRNA levels among the 37



ll lines (gray) and tumor samples (black), both
tile) and averages (diamonds; Y-value error bars
(tumor/normal ratio < 0.8), no change (0.8–1.2),
ing averages between adjacent classes (moving
umors). (b) Distribution of correlations between
bserved versus expected correlation coefficients.
The line of unity is indicated. (c) Percent variance
tained (black line) and fraction of data retained
toff values. Fraction of data retained is relative
of variation in gene expression attributable to
on of Variation in Gene Expression Attributable

though the DNA microarrays used in our
lias toward characterized and/or highly
e we are examining such a large fraction
imately 20% of all human genes), and
ove, we are likely underestimating the
opy number changes to altered gene
ur findings are likely to be generalizable
still be remarkable if only applicable to
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euploidy has been shown to result in
expression biases (13). Two recent
amine the global relationship between
gene expression in cancer cells. In
ings, Phillips *et al.* (14) have shown that
morigenicity in an immortalized pros-
, new chromosomal gains and losses
ly significant respective increase and
expression level of involved genes. In
5) recently reported that in metastatic
of genes within amplified regions were
-fold) expressed, when compared with
n. This report differs substantially from
highly amplified genes in breast cancer
creased expression. These contrasting
thodological differences between the

of expressed genes, even within existing
ion data sets, may permit the inference
rration, particularly aneuploidy (where
averaged across large chromosomal
supporting information). Fifth, this
substantial portion of the phenotypic
tension, the heterogeneity in clinical
ts' tumors may be traceable to underly-
py number. Sixth, this finding supports
pread DNA copy number alteration in
beyond the amplification of specific
of specific tumor suppressor genes.
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gene expression, might disrupt critical
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sion. Finally, our findings suggest the
rapies that exploit specific or global
ession in cancer.

ers of the P.O.B. and D.B. labs for helpful
Howard Hughes Medical Institute Physician
a portion of this work. P.O.B. is a Howard
Associate Investigator. This work was
e National Institutes of Health, the Howard
the Norwegian Cancer Society, and the
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Editorial:

Editorial Board Member: Current Biology
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Refereed papers:

1. Gertler, A., Ashkenazi, A., and Madar, Z. Binding sites for human growth hormone and ovine and bovine prolactins in the mammary gland and liver of the lactating cow. *Mol. Cell. Endocrinol.* 34, 51-57 (1984).
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6. Ashkenazi, A., Vogel, T., Barash, I., Hadari, D., Levanon, A., Gorecki, M., and Gertler, A. Comparative study on in vitro and in vivo modulation of lactogenic and somatotropic receptors by native human growth hormone and its modified recombinant analog. *Endocrinology* 121, 414-419 (1987).
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13. Ashkenazi, A., Ramachandran, J., and Capon, D. Acetylcholine analogue stimulates DNA synthesis in brain-derived cells via specific muscarinic acetylcholine receptor subtypes. *Nature* 340, 146-150 (1989).
14. Lammare, D., Ashkenazi, A., Fleury, S., Smith, D., Sekaly, R., and Capon, D. [illegible] and gp120-binding domains of CD4 are distinct and separable-

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